Nanoparticle Tracking Analysis of Cell Exosome and Nanovesicle Secretion

Simon J. Powis, Chin Y. Soo, Ying Zheng, Elaine C. Campbell and Andrew Riches
School of Medicine, University of St Andrews, Scotland

INTRODUCTION

Viruses pose a unique problem to the immune system. They reside and replicate inside cells, so are essentially hidden from immune effector molecules such as antibodies. To enable the detection and targeting of infected cells, the immune system uses a set of proteins called major histocompatibility complex (MHC) class I molecules. These function by binding small degraded viral peptides (generated as part of the normal turnover of proteins inside cells), and displays them at the cell surface to T lymphocytes. In essence, the MHC class I molecules display a ‘peptide-library’ of the cells’ internal components to the immune system. Specific anti-viral T lymphocytes can thus detect and kill a virally infected cell (because only it will be producing the viral peptide), whilst leaving adjacent healthy cells alone. It is a highly efficient and sensitive mechanism to ensure host survival in a complex pathogenic environment.

Many cells, including those of the immune system, secrete small vesicles called exosomes or nanovesicles, with a size range of between 50 and 150 nm. These vesicles can display a wide range of immunological functions, including both immunostimulatory and immunosuppressive activities. The possibility of rapidly monitoring the presence and relative quantity of exosomes in both tissue culture supernatants and body fluids by the technique of nanoparticle tracking analysis (NTA) may represent a significant step forward in the characterisation of exosomes.

KEYWORDS
light microscopy, light scattering, nanoparticle tracking analysis, exosomes, nanovesicles, MHC class I, immunology

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AUTHOR DETAILS
Dr Simon J. Powis
School of Medicine,
University of St Andrews,
St Andrews, Fife KY16 9TF, Scotland, UK
Tel: +44 (0) 1334 463 627
Email: sjp10@st-andrews.ac.uk

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PRINCIPLE OF NANO PARTICLE TRACKING ANALYSIS

NTA is a light-scattering method for nanoparticle analysis. It is being increasingly used for determining nanoparticle size through simultaneously tracking and analyzing the trajectories described by a number of individual nanoparticles undergoing Brownian motion in a fluid.

The technique is centred on a sample analysis module which comprises a small metal
housing containing a solid-state, single-mode laser diode (<30 mW, 635 nm) configured to launch a finely focused beam through the sample of liquid containing a dilute suspension of nanoparticles placed directly above a specially designed optical flat. The sample chamber is approximately 250 µl in volume and 500 µm deep and the sample is introduced by syringe via a Luer port. The sample is allowed to thermally equilibrate for 20 seconds prior to analysis.

The beam is caused to refract at the interface between the liquid sample and the optical element through which it is passed such that it describes a path which is close to parallel to the glass-sample interface (Figure 2).

Particles resident in the beam (which is approximately 100 µm wide by 25 µm deep), are visualized by a conventional optical microscope aligned normally to the beam axis and which collects light scattered from each and every particle in the field of view. A video of typically 20-60 seconds duration is taken (at 30 frames per second) of the moving particles (Figure 3). The video is analyzed by a proprietary analysis program on a frame-by-frame basis, each particle being identified and located automatically and its movement tracked. The thresholds for particle identification can be user adjusted, as can the gain and shutter speed settings of the camera, thus allowing the user to optimize the image for a particular sample type. The 8-bit video sequence can be automatically or user-adjusted in terms of image smoothing, background subtraction, setting of thresholds, removal of blurring, etc., to allow particles of interest to be tracked without interference from stray flare or diffraction patterns which can occasionally occur with non-optimum sample types.

Particles diffusing into the scattering volume are identified and followed for the duration of the particle presence in the beam or until they diffuse to within a certain distance of an adjacent particle at which point tracking ceases eliminating the possibility of analyzing particle trajectories which cross behind each other (Figure 4). Movements of individual particles are followed through the video sequence and the mean squared displacement determined for each particle. From these values, the diffusion coefficient and hence sphere-equivalent, hydrodynamic radius can be determined using the Stokes-Einstein equation and with the results shown as a particle size distribution plot (Figure 5).

M A T E R I A L S  A N D  M E T H O D S
Exosome and Nanovesicle Isolation
For exosome isolation, cells were grown in serum free medium. Culture supernatants were cleared of cells by centrifugation at 300 × g for 10 minutes, then 10,000× g for 15 minutes to remove debris. At this point culture supernatants were analysed by nanoparticle tracking analysis as described below, or further spun at 100,000× g for two hours to pellet exosomes and nanovesicles. Pellets were processed for analysis by SDS-PAGE, immunoblotting and electron microscopy as...
previously described [4]. Transmission electron microscope images were produced by Dr Alan Prescott, University of Dundee.

**Nanoparticle Tracking Analysis**

Samples were analysed using the Nanosight LM10 system (NanoSight Ltd., Amesbury, UK). Approximately 0.3 ml of sample was loaded in the sample chamber and 30-60 second videos recorded. Three videos of multiple samples were normally recorded. Both NTA v2.1 and v2.2 software were used in sample analysis.

**RESULTS AND DISCUSSION**

Established methods for the analysis of exosomes and nanovesicles include electron microscopy of ultracentrifuged (100,000×g) samples, which often display a cup-shaped morphology, as displayed in Figure 1. However, electron microscopy is limited in its capacity to rapidly determine quantitative differences in exosome secretion in multiple samples. Flow cytometry may be used, but the small vesicle sized involved is at, or approaching, the limit of most current flow cytometry systems. We have determined the ability of NTA to monitor exosome and nanovesicle secretion from a variety of cell lines and primary cells.

NTA using the LM10 setup allows for very rapid preliminary screening of samples prior to video capture and analysis. Blank control samples comprising phosphate-buffered saline or unused tissue culture media can be checked for the presence of unwanted signals, and samples for analysis can likewise be checked to determine if dilution is required. In a typical run, samples of post-20,000×g tissue culture supernatants from T-cell leukaemia lines such as Jurkat or CEM reveal the presence of a population of vesicle-like material with a typical size range of 80-120 nm (Figure 6 a and b). As confirmation that the signal obtained by NTA comprises exosome vesicles, the same sample can be subsequently centrifuged at 100,000×g and analysed by immunoblotting for the presence of typical exosomal markers such as Alix, Tsg101 and CD63.

A simple dilution of tissue culture samples also indicates that semi-quantitative data can be obtained, therefore NTA is a suitable tool for the screening of drugs and compounds that either inhibit or stimulate exosome and nanovesicle secretion (Figure 7a). To demonstrate this ability, we treated cells with monensin [5], a known potentiator of exosome activity (Figure 7b). In a similar manner, the timecourse of nanovesicle release in untreated and treated cells can be obtained. We have also used NTA to test for the stability of exosomes after multiple freeze/thaw cycles of samples and to determine the aggregation and clumping of exosomes after ultracentrifugation and resuspension in different solutes. This latter ability, to rapidly assess the monodispersity of the sample, is likely to become relevant to the wide variety of biological activities currently ascribed to exosomes and nanovesicles. Adding aggregated clumps of material may not represent their true in-vivo activity. NTA can provide reassurance of the quality of a preparation prior to further analysis.

**CONCLUSIONS**

The use of NTA in the study of exosomes and nanovesicles is still in its relative infancy. Challenges still remain: for example, as more groups use the technology and report their findings a standardisation process for sample processing, analysis and reporting will likely occur. And the technology may also be developed further.

Significant future advances will probably include the ability to record samples in one or more fluorescent modes, combined with specific labelling of subpopulations of exosomes and nanovesicles with antibodies and quantum dots. Once this is achieved it is possible to envisage NTA being used to detect a range of exosome-associated disease specific markers from serum and urine samples.

**REFERENCES**


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